Binding interactions of collagen I, laminin and fibronectin with immobilized *Escherichia coli* O157:H7 using a surface plasmon resonance biosensor

Introduction

Escherichia coli O157:H7 is an enterohemorraghic pathogen that has caused many outbreaks of foodborne illness linked to the consumption of undercooked contaminated ground beef (Bean *et al.* 1997; Bell *et al.* 1994; Council for Agricultural Science and Technology, 1994). Outer surfaces of the beef carcass can be contaminated with *E. coli* O157:H7 after slaughter. This surface (fascia) is composed of the connective tissue that lies between the skin and skeletal muscle and can be the site for bacterial adhesion. Extracellular matrix (ECM) proteins such as collagen I, laminin, fibronectin and the glycosaminoglycans (GAGs) are major components of these tissues. It is necessary to understand the interactions of the bacterial surface molecules with macromolecules of the fascia or connective tissues to elucidate the mechanisms of bacterial attachment to animal tissues.

A surface plasmon resonance biosensor, the BIAcore, allows a direct real-time detection of the binding, association and dissociation rates of the reactions without chemical labeling for signal generation (Medina, 1997). The BIAcore had been utilized to study interactions of ECM macromoleculess with bacterial cell surface adhesins. The attachment of collagen-binding adhesins of *Staphylococcus aureus* with cartilage and other host tissues were reported Patti *et al.* (1993; 1995). House-Pompeo *et al.* (1994) reported the binding interactions of collagens I-VI and fibronectin with recombinant forms of collagen binding surface adhesins and the binding kinetics of recombinant fibronectin adhesins from gram positive bacteria. Holmes *et al.* (1997) also showed that the immobilized fibronectin bound with different fibronectin binding proteins from *S. aureus* and *Staphylococcus epidermidis*. A suface plasmon resonance biosensor has not been utilized to study the binding interactions of extracellular matrix proteins and E. coli cell surface macromolecules.

We reported the use of the BIAcore to determine the binding properties of immunoglobulin (IgG) with the surface molecules of immobilized *E.coli* O157:H7 cells (Medina *et al.* 1997). This study demonstrated the feasibility of using the surface antigens of immobilized whole cells as ligands to determine the apparent association and dissociation rate constants of anti-*E. coli* IgG. This research provided a model system to study the binding interactions between the extracellular matrix proteins and the binding molecules of the immobilized *E. coli* cells.

In this study, we determined the binding characteristics of collagen I, laminin, fibronectin, hyaluronic acid and chondroitin sulfate with immobilized *E. coli* O157:H7 cells, effects of NaCl and calcium ion on binding, minimum concentration of guanidine-HCl which could effectively regenerate the sensor surface, and the apparent association and dissociation rates and binding constants of collagen I and laminin. Results obtained from this research will be utilized to study bacterial binding to connective tissues and will help design methods to control contamination of meat surfaces with pathogens.

Materials and methods

Culture and preparation of cells

E. coli O157:H7 88.1558 (*E. coli* Reference Center, University Park, PA) was maintained on tryptic soy agar (Difco laboratories, Detroit, MI). The bacteria were grown in nutrient broth (Difco) at 37°C with aeration. Five ml of cultures grown at 5, 8, and 24 h were transferred to a 15 ml conical polypropylene tube, centrifuged at 2000*g* for 10 min. The supernatant fluid was aspirated and the packed cells were resuspended in 5 ml of either 0.1M phosphate buffer (pH 7.2) or 10 mM sodium acetate buffer (pH 4). A 100 μl of bacterial suspension was transferred to microtiter wells and the optical density (OD) was measured at 405 nm. Cell suspensions with 0.7–0.9 OD (approx. 1×10^9 cfu/ml) were utilized for immobilization.

BIAcore analysis

These studies were performed on the BIAcore 1000 equipped with a BIAlogue command software. The guidelines from the manufacturer (Biosensor, Inc. Uppsala, Sweden) were followed for the preparation of the sensor surfaces, binding, interpretation of the sensorgrams and kinetic evaluation using the BIAevaluation 2.1.

Immobilization

The dextran surface of the flow cells (on the sensor chip was activated with 10 μl of a mixture of equal volumes of *N*-hydroxysuccinimide (NHS, 115 mg/ml) and *N*-ethyl-*N* = -(dimethylaminopropyl) carbodiimide (EDC, 750 mg/ml), followed by the injection of the bacteria. Aliquots (15 μl) of 5, 8 and 24 h bacterial cell suspensions were immobilized on the surface of Flow Cells 1, 2, and 3, respectively. The remaining activated dextran, not covalently bound with the cell surface, was blocked with 10 μl ethanolamine. The reagents (EDC-NHS, bacteria, ethanolamine) were injected at a flow rate of 1 μl per min. Hepes/buffered saline (HBS) pH 7.4, containing 10 mM Hepes, 3.4 mM EDTA, 0.15 M NaCl and 0.005% BIAcore P20 (v/v) was utilized as the running buffer.

Macromolecular binding and regeneration

Sodium phosphate buffer (0.1 M, pH 7.2) was utilized as dilution buffer and binding (running) buffer. Guanidine (0.5, 0.75, 1.0, and 2 M) was acidified with HCl to pH 2.5 and the minimum concentration which can effectively regenerate the sensor surface was determined. A chemically characterized collagen I (Chang *et al.* 1996) was used in this study and was a gift from Dr. Paul Chang (Matrix Pharmaceutical, Fremont, CA). Laminin, fibronectin (bovine plasma), hyaluronic acid from bovine trachea and chondroitin sulfate A were obtained from Sigma. Collagen I, laminin and fibronectin were analyzed at 50 or 100 μg/ml while hyaluronic acid and chondroitin sulfate (100 and 400 μg/ml, respectively) were injected in a volume of 20 or 30 μl with a flow rate of 2 μl/min. The sensor surface bound with analytes was regenerated by using 2 pulses (1 μl each) of 0.75M guanidine·HCl, pH 2.5. Binding interactions of 10 mM calcium chloride or 0.15 M sodium chloride added to 10 mM phosphate buffer were observed. Phosphate buffer (10 mM) without $CaCl_2$ or NaCl was used as running buffer.

Collagen I, laminin, and fibronectin were mixed as binary or ternary mixtures prior to the BIAcore analysis. Four different sensor chips were immobilized with *E. coli* and utilized in these binding studies. Binding interactions of the extracellular matrix components with cell surfaces of the 5, 8 and 24 h cultures were observed. Phosphate buffer (0.1 M, pH 7.2) was used as dilution and running buffers. The kinetic values of dissociation and association rate constants were determined using BIAcore's BIAevaluation software.

Results and discussions

Extracellular membrane macromolecules

Chondroitin sulfate and hyaluronic acid exhibited no binding with the bacterial sensor surface even at 400 μg/ml. However, all concentrations of laminin and collagen I bound to the immobilized cell surfaces. Sensorgrams showing typical binding responses of the ECMs are shown in Figure 1. Laminin (100 μg/ml) binding generated 1395, 1251, and 1145 Response Units with 5, 8 and 24 h cultured cell surfaces, respectively. Speziale *et al.* (1982) described laminin binding with the hair-like structures (pili) of uropathogenic *E. coli*, but, laminin did not bind with the *S. aureus* surface. Collagen I (50 (g/ml) binding

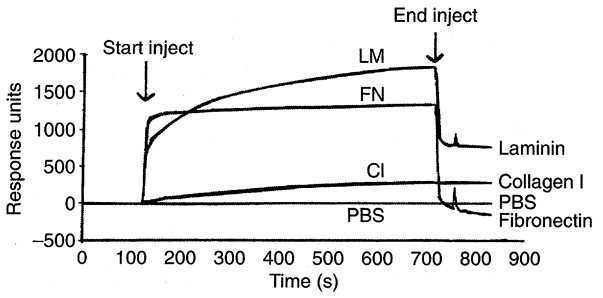

Figure 1 An overlay sensorgram showing the binding of 200 nM fibronectin (FN), 125 nM laminin (LM), and 172 nM collagen I (CI) vs PBS (phosphate buffer). The sensor surface was immobilized with *E. coli* which had been cultured for 8 h. RU between the start and end of sample injection indicate the total molecular mass detected by the photodiode array but the captured molecular mass after sample injection is indicated as bound molecules.

Table 1 Binding responses of fibronectin, laminin and collagen I with the immobilized *E. coli 0157:H7* cells and their apparent association and dissociation rates.

Flow cell no. (Sensor RU)	*Culture, h (optical density)*	*Sample injected*	*RU bound (RU desorbed)*	*Dissociation rate, k_d (s^{-1})*	*Association rate k_a (M^{-1} S^{-1})*
1 (3RU)	5 (0.70)	Fibronectin 100 µg/ml (200 nM)	(–180) (–34) (–635)		
		Laminin 100 µg/ml (125 nM)	1395 1201 884	1.17×10^{-4} 8.64×10^{-4} 4.69×10^{-3}	1.59×10^{4} 1.86×10^{4} -1.01×10^{4}
		Collagen I 50 µg/ml (172 nM)	1068 980 927	3.90×10^{-5} 1.62×10^{-4} 9.12×10^{-4}	1.02×10^{4} 2.18×10^{4} 1.17×10^{4}
2 (273 RU)	8 (0.972)	Fibronectin 100 µg/ml (200 nM)	91 (–10) (–101)		
		Laminin 100 µg/ml (125 nM)	1251 775 457	6.16×10^{-5} 9.42×10^{-4} 3.12×10^{-3}	1.50×10^{4} 1.41×10^{4} -1.38×10^{4}
		Collagen I 50 µg/ml (172 nM)	524 265 219	-2.78×10^{-4} -2.80×10^{-4} 1.56×10^{-3}	1.64×10^{3} 0.83×10^{3} 5.28×10^{3}
3 (269 RU)	24 (0.940)	Fibronectin 100 µg/ml (200 nM)	109 67 (–265)		
		Laminin 100 µg/ml (125 nM)	1145 641 426	7.96×10^{-4} 1.13×10^{-3} 2.19×10^{-3}	1.42×10^{4} 1.66×10^{4} 4.23×10^{3}
		Collagen I 50 µg/ml (172 nM)	488 249 486	-2.64×10^{-4} -2.95×10^{-4} 2.20×10^{-3}	1.53×10^{3} -1.04×10^{4} 1.40×10^{3}

with the 5, 8 and 24 h cultured cell surface generated 1068, 1251, and 488 RU, respectively. Fratamico *et al.* (1996) also showed binding of collagen I with this *E. coli* strain. Fibronectin had lower binding than collagen I or laminin but the binding response increased from 5 to 8 to 24 h cell surfaces (−180, 91, 109 RUs). The negative RU indicated that residual bound proteins on the sensor surface were detached by fibronectin when a 5-h cell culture was used as the sensor surface.

In this study, production of curli was prominent in the 8 h culture and increased at the 24 h culture (data not shown). However, electron micrographs of the curli produced by the same strain of *E. coli* O157:H7 88.1558 grown overnight had been shown previously (Fratamico *et al.* 1996). Olsen *et al.* (1989) also reported that coiled surface structures (curli) produced by certain *E. coli* strains had the ability to bind fibronectin and suggested the mediation of fibronectin with virulent *E. coli*. However, earlier studies by Speziale *et al.* (1982) showed no binding of fibronectin to any of the uropathogenic *E. coli* strains tested.

The sensor surfaces bound by the ECM proteins were regenerated with two 30 s pulses of guanidine·HCl (0.75M, pH 2.5) removing 85–99% of collagen I and laminin from sensor surfaces bound with individual components. When the sensor surfaces were exposed to different ECM components, complex interactions occurred, such as generation of complexes with strong affinity to the bacterial surface leaving residual proteins on the sensor surface as shown by an increase in background surface RU after guanidine·HCl regeneration. This residual bound complex blocked binding sites on the bacterial surface as shown by a decrease in collagen and laminin binding in the second and third replicate analysis (Table 1). Fibronectin mediated removal of these complexes from the sensor surface. Table 1 also shows the sensor surface characteristics and the relative apparent dissociation rates of laminin and collagen determined by the BIAevaluation system. There

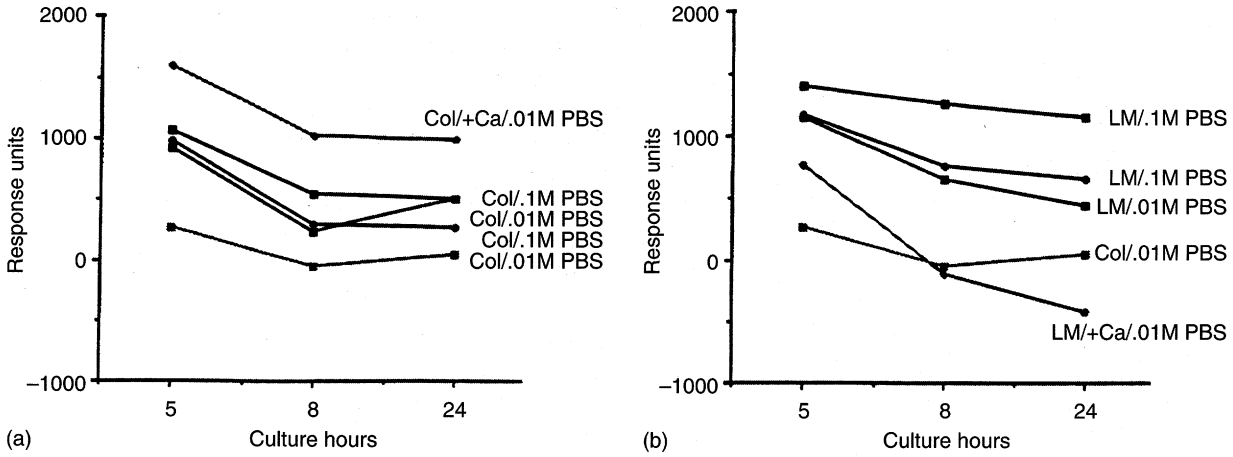

Figure 2 Interactions of 10 mM calcium chloride and 10 or 100 mM phosphate buffer (pH 7.2) with 172 nM collagen I (A) and 125 nM laminin (B).

was an apparent increase in dissociation rates (k_d values) in the 2nd and 3rd replicates. These kinetic values can be considered as preliminary kinetic values while ongoing research attempts to improve the precision and reproducibility of these binding interactions.

The ECMs were injected at a flow rate of 2 μl/min and were allowed to bind with the sensor surface for 10 min. The sensor surface was regenerated with 0.75 M guanidne·HCl, pH 2.5 (2 × 30 sec pulses). Three replicate experiments were performed using same sensor chip. Heparan sulfate was utilized in the latter 2 experiments. RU (response unit) bound is the molecular mass captured by sensor surface. The negative RU indicate desorption of some proteins that remained on the sensor surface or loss of the bacterial sensor surface. Affinity constants ($K = k_a/k_d$, M^{-1}) ranged from 10^7 to 10^9 M^{-1}.

The association rates were in the range of 10^3 to 10^4 and had greater variability with the sensor surface with which 24 h cells were immobilized. The changes and variabilities in kinetic values are presumably due to the complex interactions of the ECMs on the bacterial surface, presence of multiple binding sites on the bacterial sensor surface and the ECMs, and the large molecular mass and heterogeneity of laminin (M_r = 800,000) and collagen (M_r = 290,000). Kinetic analysis of collagen binding indicated that collagen I rebound on the sensor surface or matrix during the dissociation phase and this rebinding may have resulted in negative k_d values. The negative association rate values of laminin were perhaps due to a large ratio of mass effect versus the binding signals which are not the ideal conditions for kinetic analysis by the BIAevaluation system. Fibronectin (M_r = 500,000) generated very low binding signals and its dissociation rates were not determined.

Ionic concentrations of 10 and 100 mM sodium phosphate buffer, pH 7.2 had no effect on ECM binding with the bacterial sensor surface (Figure 2). However, addition of 150 mM NaCl inhibited binding of all ECM proteins. Calcium chloride (10 mM) added to 10 mM phosphate buffer inhibited binding of laminin while it enhanced binding of collagen and fibronectin. Results from this study suggest that calcium mediated the binding of collagen I and fibronectin with the *E. coli* surface. The binding effects of collagen and laminin are shown in Figures 2a and 2b. Response Units generated from the interactions of calcium treated fibronectin with the 5, 8, and 24 h bacterial sensor surface were 248, 537 and 307 RUs and were −269, −393 and −539 RUs from untreated fibronectin, respectively. Again, this data suggests a calcium enhanced binding of fibronectin with *E.coli* surface.

Similar results were reported by Kramer (1994) showing that Ca^{+2} inhibited binding of laminin with integrin. In contrast, Mg^{2+} or Mn^{2+} were also required in integrin-mediated adhesion of cells to collagen. Ca^{2+} and Mg^{2+} also enhanced binding of collagen to fibroblasts (Kleinman *et al.* 1978). Calcium and collagen I interactions are also demonstrated in bone structure (Brodsky and Eikenberry, 1982) where the collagen fibrils are associated with hydroxyapatite (calcium phosphate hydroxide). Landis (1996) described the association of calcium with collagen in bones and connective tissues and further showed that calcium interacted with the telopeptide portion of collagen.

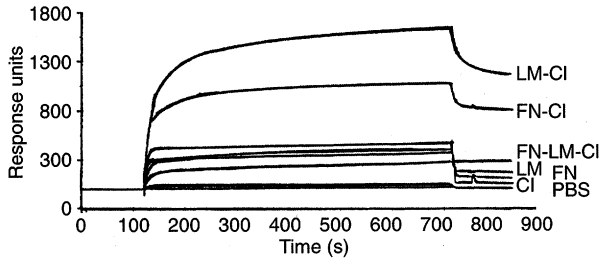

Figure 3 Synergistic binding effects of binary mixtures of collagen and the relative binding responses of collagen I (CI), laminin (LM) and fibronectin (FN) over the bacterial sensor surface immobilized with an 8 h *E. coli* O157:H7 culture. Individual and binary mixtures contained 50 μg/ml each of CI, FN and LM while the ternary mixture contained 33 μg/ml of each protein.

Binary combinations of laminin, collagen I and fibronectin showed synergistic binding effects with the bacterial sensor surface (Figure 3). Laminin-collagen and fibronectin-collagen had higher binding responses compared to the binding of the individual compounds at an equal concentration. In contrast, fibronectin in combination with laminin and collagen had inhibitory effect shown by the reduced binding response compared to binding of laminin-collagen. In Figure 3, responses generated by individual proteins were lower than previous experiments on same sensor surface. Another experiment on same sensor surface showed 286, 654, and 259 RUs generated from the binding of 33.3 μg/ml each of fibronectin-collagen, laminin-collagen and fibronectin-laminin-collagen I mixtures, respectively. The results from our studies suggest that the synergistic binding responses of the combination of laminin and collagen I mediated binding with the *E. coli* O157:H7 surface. In contrast, fibronectin in combination with laminin and collagen had an inhibitory effect shown by the reduced RUs compared with the binding of binary pairs, laminin-collagen and fibronectin-collagen.

Studies showed that fibronectin interacted with collagen (Engvall *et al.*, 1978; *Dessau et al.*, 1978; Kleinman *et al.*, 1978). Cold insoluble globulin from serum inihibited binding of CHO (Chinese hamster ovary) and fibroblast cells to collagen coated surfaces. Visai *et al.* (1990; 1991) found that fibronectin and laminin also bound to an enterogetoxigenic strain of *E. coli* and that laminin competed with collagen binding sites. Olsen *et al.* (1994) also reported that collagen I inhibited fibronectin-enhanced binding of curli expressing *E. coli* to skin. They also observed that fibronectin which bound to the curli mediated bacterial attachment to the reticular layer and suggested that collagen I in the ocular tissue acted as the binding receptor. Laminin and collagen IV network and their interaction was shown by Yurchenco and Schittny (1990) in a model system. The assembly and interactions of extracellular matrix proteins including collagen, laminin and fibronectin were reviewed by Beck and Gruber (1995). Ofek and Doyle (1994) also reviewed recent developments in adhesion characteristics of various strains of *E. coli* to animal cells and their interactions with ECMs and other compounds. Such interactions varied with different *E. coli* strains.

Cell aggregation assay

Detection of cell aggregation by optical density measurement at 405–650 nm did not clearly show differences between treatments. The 24 h cultured cells packed closely along the sides of the wells indicating greater hydrophobic attraction to the polystyrene walls compared to the 8 and 5 h cultures. The 24 h cultured cells had a dense network of pili or curli (data not shown). Fratamico *et al.* (1996) had shown the surface structures of this *E. coli* strain and its interaction with collagen.

Effects of collagen, laminin, fibronectin, hyaluronic acid and chondroitin sulfate on cell aggregation was observed through an optical microscope at 40 × magnification. At this magnification, cell aggregation was observed when the bacterial cells were treated with collagen I or laminin and aggregation was clearly detectable compared to cells suspended in PBS. Collagen I showed larger aggregates than laminin aggregates (data not shown). Effects of fibronectin were not clearly discernible compared to self aggregation of the bacterial cells. When fibronectin was combined with collagen I and laminin, there was less cell aggregation compared to effects on the cells by either collagen or laminin. Chondroitin sulfate and hyaluronic acid had no effects on *E. coli* cell aggregation. These results are in agreement with the chemical interactions shown by the BIAcore studies. Electron microscopy studies of these interactions are in progress and will be reported separately, but the preliminary data also confirms the results observed with the BIAcore and optical microscopy.

Conclusions

Real-time analysis of the binding of collagen I, laminin, fibronectin and glycosaminoglycans with immobilized *E. coli* O157:H7 cells showed the adhesive properties of the macromolecules with the bacterial surface. Laminin and collagen bound with the bacterial sensor surface and when combined, binding with the bacteria was enhanced while fibronectin reduced the binding of the mixture. Saline had inhibitory binding effects while hyaluronic acid and chondroitin sulfate had no interaction with *E. coli* O157:H7 surface. Calcium inhibited laminin binding but enhanced

collagen I binding. These interactions suggest the complex nature of attachment of tissue macromolecules with bacterial surfaces. This study demonstrates the use of the BIAcore to screen for the binding properties of unmodified macromolecules with immobilized bacterial cells. This model system will lead us to effectively screen for compounds which can inhibit or detach binding of food pathogens on carcass surfaces of slaughtered farm animals.

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